Published in final edited form as:
Int J Syst Evol Microbiol. 2019 January ; 69(1): 203–213. doi:10.1099/ijsem.0.003130.

Polynucleobacter paneuropaeus sp. nov., characterized by six strains isolated from freshwater lakes located along a 3000 km North-South gradient across Europe

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Abstract

Six *Polynucleobacter* (*Burkholderiaceae*, *Betaproteobacteria*) strains isolated from different freshwater lakes located across Europe were taxonomically investigated. Phylogenetic analyses based on 16S rRNA gene sequences assigns all six strains to the cryptic species complex PnecC within the genus *Polynucleobacter*. Analyses of partial glutamine synthetase (glnA) genes suggests that all six strains belong to the species-like taxon designated F15 in previous papers. Comparative genome analyses reveal that the six strains form a genomically coherent group characterized by whole-genome average nucleotide identity (gANI) values of > 98% but separated by gANI values of < 88% from the type strains and representatives of the 16 previously described *Polynucleobacter* species. In phylogenetic analyses based on nucleotide sequences of 319 orthologous genes, the six strains represent a monophyletic cluster that is clearly separated from all other described species. Genome sizes of the six strains range from 1.61 to 1.83 Mbp, which is smaller than genome sizes of the majority of type strains representing previously described *Polynucleobacter* species. By contrast, G+C content of the DNA of the strains is well in the range of 44.8-46.6 mol% previously found for other type strains of species affiliated with the subgroup PnecC. Variation among the six strains representing the new species is evident in a number of traits. These include gene content differences, for instance regarding a gene cluster encoding anoxygenic photosynthesis, as well as phenotypic traits. We propose to name the new species represented by the six strains *P. paneuropaeus* sp. nov. and designate strain MG-25-Pas1-D2^T (=DSM 103454^T =CIP 111323^T) as the type strain.

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Conflicts of interest
The authors declare the absence of any conflict of interest.

Ethical statement
The presented study does not include any experimental work with humans or vertebrates.

DDBJ/EMBL/GenBank accession numbers
Genome sequences of *Polynucleobacter paneuropaeus* sp. nov strains MG-25-Pas1-D2^T, MWH-UK1W16, MWH-Creno-4B4, UB-Kaiv-W7, MWH-CNW20-3, and FUKU-NW11 were deposited under the accession numbers, CP030085, QMCG00000000, CP030086, CP030088, CP030087, and QMCH00000000, respectively. Their 16S rRNA gene sequences were deposited under the accession numbers MH492629, AM110086, AM110104, MH492749, MH492624, and MH492748, respectively.

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The genus *Polynucleobacter* (family *Burkholderiaceae*, class *Betaproteobacteria*) and the species *P. necessarius* were described by Klaus Heckmann and Helmut J. Schmidt as obligate endosymbionts dwelling in ciliates affiliated with the genus *Euplotes* [1]. The symbiosis between the ciliates and the bacteria is mutually obligate, that is both partners rely on each other and cannot survive if separated [1, 2]. The obligate endosymbiotic lifestyle of these bacteria prevented their cultivation when separated from their hosts. Therefore, the type species of the genus, *P. necessarius*, is not represented by a type strain but by type material consisting of endosymbionts contained in a culture of *E. aediculatus* [1]. Unfortunately, this type material is not available anymore from the American Type Culture Collection (ATCC) or from any other culture collection [3].

Years after the description of the obligate endosymbiotic species *P. necessarius*, it was discovered by using cultivation independent methods that bacteria closely related to *P. necessarius* are abundant dwellers in the pelagic zone of freshwater systems [4–8]. Investigations by fluorescent *in situ* hybridization (FISH) with probes specific for the genus *Polynucleobacter* or for particular subgroups revealed an ubiquitous presence of the genus in standing freshwater systems and determined a variable relative abundance between systems and habitat types in the range of < 1% to 67% of total bacterial numbers [9, 10]. Importantly, those *Polynucleobacter* cells visualized by FISH in freshwater samples appeared as freely suspended cells, which suggested that these cells do not dwell as obligate endosymbionts but as free-living planktonic bacteria. Isolation of a large number of *Polynucleobacter* strains [11] from a broad variety of freshwater systems and geographic locations [12] enabled preliminary phylogenetic characterization of the genus *Polynucleobacter* and revealed the presence of at least five subclusters designated PnecA, PnecB1, PnecB2, PnecC, and PnecD [10]. Analyses by FISH suggested that subcluster PnecC is the most abundant *Polynucleobacter* subgroup is in many habitats [13]. A preliminary delimitation of subcluster PnecC in operational taxonomic units (OTU) detectable by a cultivation-independent method in environmental samples revealed OTU-specific ecological preferences and suggested that ubiquity of subcluster PnecC in freshwater systems resulted from ecological diversification among PnecC lineages [14]. Interestingly, strains affiliated with subcluster PnecC usually share 16S rRNA gene sequence similarities > 99%. Nonetheless, it was recognized that subcluster PnecC harbours a large number of species that cannot be discriminated by 16S rRNA sequences [15]. Thus, subcluster PnecC represents a large cryptic species complex if only 16S rRNA sequences are consulted. Ten free-living and one endosymbiotic species are currently described. The other four *Polynucleobacter* subclusters currently contain five described free-living species [16–20].

Here, we aim for the taxonomic description of a new *Polynucleobacter* species, representing a taxon that is affiliated with subcluster PnecC and has been previously designated F15 [14]. In a population genomics study about this taxon, aiming for insights into the intraspecific diversity (Hoetzinger and Hahn, in prep.), a total of 119 affiliated were isolated from 53 habitats scattered across Europe. The majority of strains were obtained by a combination of high-throughput cultivation and molecular screening for cultures containing F15 strains. In total 113 of the obtained strains have been genome sequenced. Analyses of pairwise whole-genome average nucleotide identities (gANI) suggested that all 113 strains belong to a single

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species. For taxonomic characterization in the present paper, we selected six strains that represent the maximum geographic and climatic gradient covered by the 113 isolated strains. We characterize all six strains genomically, and three of them further phenotypically and chemotaxonomically. Based on genomic and phylogenetic analyses the six strains represent a coherent group well separated from the type strains of previously described *Polynucleobacter* species. The commonly accepted threshold of 95-96% gANI for species demarcation in prokaryotes [21] suggests that the six strains constitute a new *Polynucleobacter* species. We propose to establish the new species *P. paneuropaeus* sp. nov. for these strains.

Home Habitats and Isolation

The six investigated strains were isolated from freshwater lakes scattered across Europe (Table 1, Fig. 1). The latitudinal distance between the most northward and the most southward habitat is 3028 km and the geographic distance between these two locations is 3260 km. The six ecosystems include a lake located in the boreal zone north of the polar circle, a boreal lake located south of the polar circle, three lakes located in Central Europe, one of which is located at an altitude of 2116 m in the Austrian Alps, and a lake located on the Mediterranean Island Corsica. The six lakes are characterized by pH values in the range of 4.6 – 7.5 and conductivity in the range of 15.4 – 55.4 μS cm^{-1} (Table 1). The relatively low conductivity values result from the geology in the catchment area of the lakes, primarily silicate bedrock but lacking limestone. Consequently, concentrations of dissolved calcium and magnesium carbonates are low in all six lakes and the water is characterized as soft. By contrast, the lakes differ in concentrations of humic substances. While Lake Unterer Klaffersee represents a clear water lake with low concentrations of humic substances and high transparency, other lakes represent humic bog lakes with brown stained waters.

All six strains were isolated by using the filtration acclimatization method [22] without molecular screening as described previously [11]. NSY medium [22] was used for isolation and maintenance of the strains. Soon after the establishment of pure cultures, the strains were cryopreserved at -70°C in NSY medium supplemented with 15% glycerol.

Genomic Characterization

DNA extraction was performed for all six strains as described previously for other *Polynucleobacter* type strains [23–25]. Genome sequencing and assembly for strains MG-25-Pas1-D2^T, MWH-Creno-4B4, MWH-CNW20-3, and UB-Kaiv-W7 has been performed as described previously for the type strain of *Silvanigrella aquatica* [26]. Two DNA libraries were constructed for each strain. A shotgun library was paired-end sequenced by a Roche GS FLX instrument (Titanium chemistry) and a Long Jumping Distance (LJD) library of 8 kb fragment size was mate-pair sequenced on an Illumina MiSeq instrument (2 x 150 bp). A de novo hybrid assembly combining both types of libraries was conducted by Eurofins Genomics using their in-house pipeline based on the software tool newbler 2.9. This approach resulted in closed genomes for all four strains, which are illustrated in the Supplementary Materials Fig. S1. For strains MWH-UK1W16 and FUKU-NW11 shotgun libraries were paired-end sequenced by a Roche GS FLX instrument (Titanium chemistry)

and an Illumina MiSeq instrument (2 x 300 bp), respectively. Draft genomes consisting of seven and fourteen scaffolds were obtained for these two strains (Table 2).

Genome sizes of the six new strains range from 1.61 to 1.83 Mbp, i.e. are smaller than those of all previously characterized type strains of free-living species affiliated with subcluster PnecC. The only exception among described PnecC species is strain STIR1, representing the obligately endosymbiontic *P. necessarius*. Eight further genomes of obligately endosymbiotic strains affiliated with PnecC are available, with sizes ranging from 1.55 to 1.93 Mbp [27]. Thus, genome sizes of the six free-living strains investigated here are smaller than those of certain endosymbiotic *Polynucleobacter* strains. However, the endosymbiotic strains are characterized by relatively high numbers of pseudogenes [27]. Among the five *Polynucleobacter* species not affiliated with subcluster PnecC, the *P. cosmopolitanus* (1.78 Mbp) and *P. victoriensis* (1.63 Mbp) type strains exhibit genome sizes within the range of the six investigated F15 strains [28]. On the other hand, G+C values of the genomes of the six new strains (45.6-46.1%) fall well within the narrow range previously reported for the other described species of subcluster PnecC (Table 2) and are higher than those of the *P. cosmopolitanus* (44.1%) and *P. victoriensis* (43.1%) type strains.

All six new strains encode putative FeoAB Fe^{2+} transporters but lack genes putatively encoding ABC-type Fe^{3+} transporters (Table 3), which suggests an adaptation to acidic or circum-neutral pH conditions but a lack for adaptation to alkaline conditions [15]. Interestingly, all six strains possess genes putatively encoding a cytochrome bd-I terminal oxidase, which suggests adaptation to low oxygen concentrations [29]. The six strains differ, for example, in the presence of genes putatively enabling utilization of nitrate, nitrite or cyanate as inorganic nitrogen sources, in the presence of gene clusters putatively encoding an apparatus for anoxygenic photosynthesis, or genes for synthesis of flagella (Table 3).

Average nucleotide identity analyses [30] based on whole genome sequences (gANI) performed with the Integrated Microbial Genomes (IMG/M ER) system [31] suggest only small average sequence differences among genes shared by all six strains. The average value of all pairwise gANI comparisons among the six strains is 98.4% with a minimum value of 98.1% (MG-25-Pas1-D2^T vs.UB-Kaiv-W7) and a maximum value of 98.8% (MWH-Creno-4B4 vs. MWH-UK1W16). The obtained gANI values are based on alignments representing 87% of the investigated genomes on average (range of alignment fractions 82-94%). In contrast to the high gANI values among the strains investigated here, they all share < 80% gANI with the type strains of all previously described *Polynucleobacter* species. Sequence similarity data for comparisons with strain MG-25.Pas1-D2^T are given in Fig. 2.

The genome of MG-25-Pas1-D2^T reveals a large rearrangement compared to the three other closed genomes (Fig. 3), although the syntenies of the four closed genomes are reliable due to the hybrid assemblies involving reads from the 8 kb LJD library. In the MG-25-Pas1-D2^T genome, the region ranging from 0.38 – 1.39 Mbp after the origin of replication (*ori*) is present as reverse complement when aligning the region around the *ori* to the other genomes. Vice versa, the region around the *ori* (0.39 Mbp before – 0.29 Mbp after) is reversed when the rest of the genome is oriented according to the three reference genomes. The inversion is

asymmetric relative to the *ori*, similar as illustrated in Figure 3b in [32]. The transition regions between the inverted chromosome segments are marked by two genomic islands, i.e. regions of 92 kbp and 54 kbp, respectively, without homology to the reference genomes (Fig. 3 and Supplementary Materials Fig. S1). However, these genomic islands share a sequence of 313 bp (94% nucleotide sequence similarity) that includes a transposase (IMG Locus Tags Ga0256622_11348 and Ga0256622_111498). This suggests that the inversion, which likely happened in an ancestor of strain MG-25-Pas1-D2^T, has been mediated by replicative transposition.

Phylogeny

According to 16S rRNA gene sequences, the six investigated strains are affiliated to the cryptic species complex PnecC [3] within the genus *Polynucleobacter*. This assignment is also confirmed by the presence of a PnecC-specific signature sequence in the 16S rRNA gene of all six strains [3]. However, reconstruction of the phylogenetic positions with a suitable resolution is not possible by using 16S rRNA gene sequences (Supplementary Materials Fig. S2). A much better phylogenetic resolution was obtained by analyses based on an alignment of 319 shared genes as described previously [28]. Briefly, nucleotide sequences of 319 genes shared by all *Polynucleobacter* type strains and *Cupriavidus metallidurans* CH34^T were extracted from genome sequences and aligned by using the software MAFFT [33]. This resulted in a total alignment length of 342,064 bp. The software GBlocks Masking 3.9.17 [34] was used to select conserved blocks from the alignment for the further analyses. This resulted in 303,752 positions (88%) in 701 selected blocks. The CIPRES Science Gateway V. 3.3 [35] was used to calculate a bootstrapped (100 resamplings) RAxML tree [36] (Fig. 2). In accordance to the 16S rRNA gene phylogeny, this tree based on a large multi-gene alignment also places the six strains in subcluster PnecC but as a tight cluster only consisting of the six new strains, which is well separated from all other taxa. This tree suggests for taxon F15 the most basal position of all PnecC species investigated so far. Yet, the reconstructed position of taxon F15 is closer to all investigated members of subcluster PnecC than to all previously investigated *Polynucleobacter* taxa not affiliated with PnecC.

Phenotypic and Chemotaxonomic Characterization

The phenotypic and chemotaxonomic characterization of three strains representing taxon F15 was performed as described previously [28, 37]. Because of the inferred phylogenetic distances between the six new strains and the previously investigated type strains (Fig. 2), the investigated strains were compared with all previously described PnecC taxa. The obtained results are presented in Tables 4 and 5. As with many other *Polynucleobacter* strains, the investigated strains formed small circular, convex, and colourless colonies with shiny surface on NSY agar plates [22]. In contrast to all type strains affiliated with subcluster PnecC, all three investigated new strains assimilated both glyoxylate and glycolate (Table 4). Among the five type strains of *Polynucleobacter* species not affiliated with subcluster PnecC only the *P. rarus* type strain showed assimilation of both substances but only weak assimilation of glycolate [17]. Although the assimilation of glyoxylate and glycolate represents a phenotypic trait tentatively discriminating the three strains from other

PnecC species, we refrain from over-interpretation of these data. *Poylnucleobacter* isolates are relatively weakly growing in lab cultures (Supplementary Fig. S2 in [38]), and relatively unreliable and slow in adjusting their metabolism, presumably due to remarkably low numbers of signal transduction genes [39]. For example, differences between expectations from gene content and phenotype observed for *Polynucleobacter* bacteria earlier have been attributed to lack of gene expression under certain cultivation conditions [40]. It is possible that certain substrates for which no utilization has been found could be assimilated by the respective strain after excessive acclimatization. In summary, the volatile growth of *Polynucleobacter* in culture may be responsible for some of the observed differences in substrate assimilation patterns. Therefore, it is not practicable to consult substrate assimilation patterns for discrimination between *Polynucleobacter* species.

The analysis of the whole-cell fatty acid composition (Table 5) was carried out as described previously [19]. The cell masses were cultivated on R2A [41] agar slants which were filled up with 1.5 ml liquid R2A medium at 28°C. The slants were inspected for growth daily. Once biomass was well visible at the lowest point of the slope, the cell mass was harvested. The incubation periods were 6 days for strains MG-25-Pas1-D2^T and MWH-Creno-4B4, and 7 days for strain MWH-UK1W16. As for all strains of the PnecC complex tested so far, the fatty acids were dominated by $C_{16:1}$ ω7c and $C_{16:0}$. Noticeably, the variation of the percentages of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ ω7c, 11-methyl, and $C_{18:1}$ ω7c was high among the three F15 strains. There is no specific fatty acid composition feature that differentiates the F15 strains from others of the PnecC complex.

Proposal of the New Species *Polynucleobacter paneuropaeus* sp. nov.

The performed phylogenetic and gANI analyses clearly suggest that the six investigated strains are closely related to each other and represent a genomically coherent group of bacteria characterized by gANI values of > 98% but separated from all previously established *Polynucleobacter* type strains by gANI values of < 80%. Commonly, a threshold of 95-96% gANI is accepted for species demarcation in prokaryotes [21], which clearly suggests that the six strains represent a new *Polynucleobacter* species.

All three phenotypically characterized strains can be discriminated from all previously established *Polynucleobacter* type strains by their ability to assimilate both glyoxylate and glycolate (Table 4). The only exception is the type strain of *P. rarus*, however, all six genomically characterized new strains can be discriminated from the *P. rarus* type strain, for instance, by the G+C values of their DNA, which was 45.6 – 46.1 mol%, while the value of the *P. rarus* type strain was 40.3 mol%. While the six new strains could not be discriminated by comparative analyses of 16S rRNA gene sequences from type strains of previously described *Polynucleobacter* species, comparison of partial glnA sequences [42] enabled an efficient sharp-cut (> 99% versus < 90%) discrimination from other strains (Fig. 2).

We propose to establish the new species *Polynucleobacter paneuropaeus* sp. nov. for taxon F15, represented by the six strains investigated here. We designate strain MG-25-Pas1-D2^T (=DSM 103454^T =CIP 111323^T) as the type strain.

Description of *Polynucleobacter paneuropaeus* sp. nov.

Polynucleobacter paneuropaeus (pan.eu.ro.pae'us. N.L. masc. adj. *paneuropaeus* pan-European, dwelling all over Europe in suitable freshwater systems).

Contains free-living *Polynucleobacter* strains dwelling in the water body of acidic and circum-neutral freshwater systems. Cells are short rods, 0.4-1.2 μm in length and 0.3-0.6 μm in width, depending on cultivation conditions. Aerobic and chemoorganoheterotrophic, weak anaerobic growth was observed in one strain. Colonies grown on NSY agar are non-pigmented, circular and convex with smooth surface. Growth occurs up to 31-32 °C. Growth occurs in the presence of 0-0.4% (w/v) NaCl, one strain weakly grows at 0.5% (w/v) NaCl. Utilizes glyoxylate, glycolate, acetate, pyruvate, oxaloacetate, malate, fumarate, succinate, and L-cysteine. All strains weakly utilize D-mannose. Do not utilize D-glucose, D-galactose, L-fucose, D-sorbitole, L-histidine, L-aspartate, L-alanine, L-asparagine, L-leucine, L-serine, and betaine. Strains vary in the utilization of propionate, malonate, oxalate, citrate, levulinate, D-galacturonate, D-Lyxose, D-fructose, and D-glutamate. Major fatty acids are $C_{16:1}$ ω7c, $C_{16:0}$, $C_{18:1}$ ω7c, and feature 2 containing $C_{16:1}$ isoI and $C_{14:0}$-3OH. Encodes a cytochrome bd-I terminal oxidase, a FeoAB Fe^{2+} transporter but no ABC-type Fe^{3+} transport system. Some but not all strains encode a putative system for anoxygenic photosynthesis. Genome sizes range from 1.61 – 1.83 Mbp and G+C content of the DNA ranges from 45.6 – 46.1 mol%. The type strain is MG-25-Pas1-D2^T (=DSM 103454^T =CIP 111323^T), isolated from Lake Russevatn in Norway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Gabriele Pötter for carrying out the fatty acid analyses.

Funding information

This study was supported by the Austrian Science Fund (FWF) project 27160-B22.

Abbreviations

FAM	filtration acclimation method
FL	free-living
E	endosymbiotic
gANI	whole genome average nucleotide identity
IMG/M ER	Integrated Microbial Genomes with Microbiome Samples Expert Review companion system
kbp	kilo base pairs
LJD	Long Jumping Distance

Mbp	mega base pairs
ML	maximum-likelihood
NSY medium	nutrient broth soytone yeast extract medium
OD	optical density
ori	origin of replication
PnecC	*Polynucleobacter* subgroup PnecC (cryptic species complex)
R2A medium	Reasoner's 2A medium

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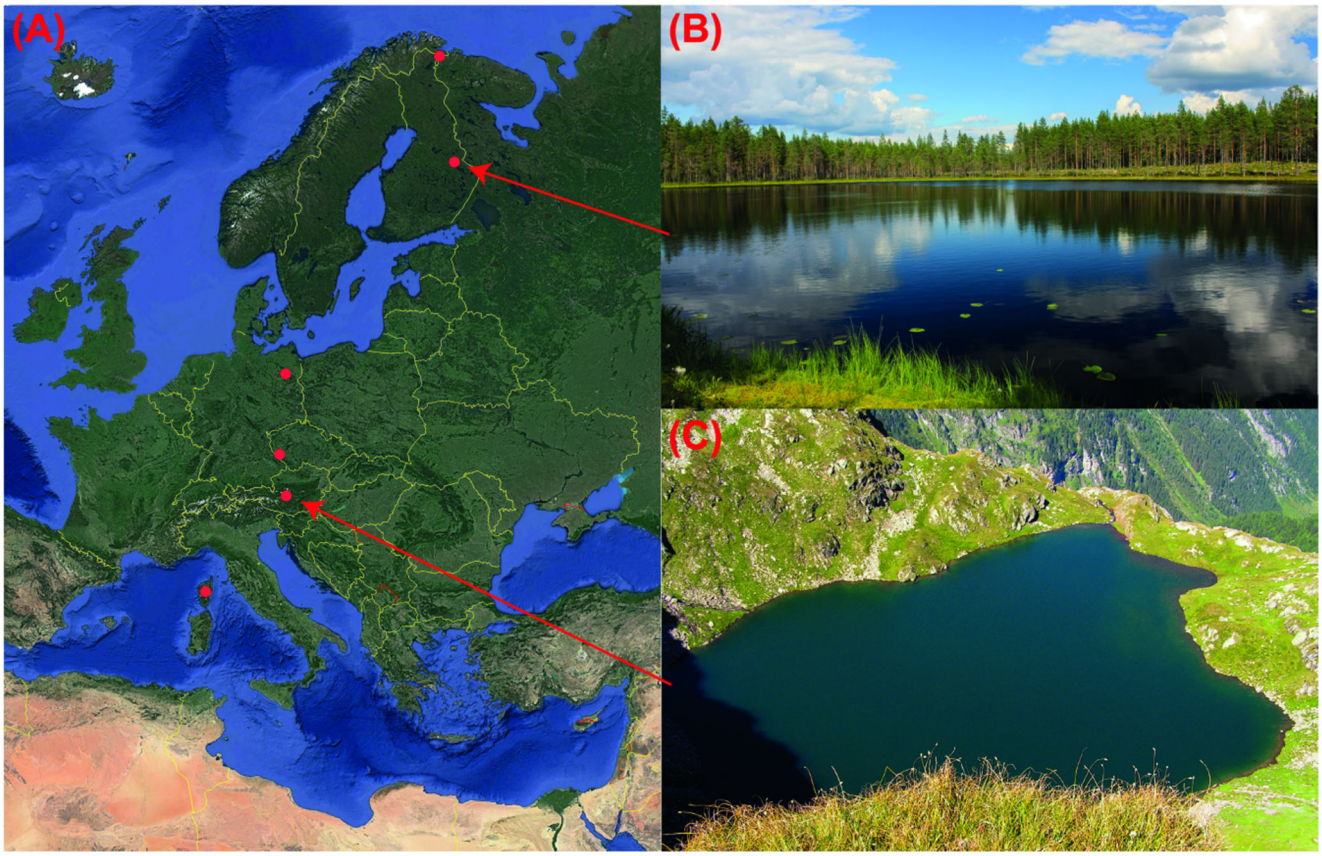

Fig. 1.

(A) Locations of the lakes from which the six investigated *P. panaeropaeus* sp. nov. strains were obtained. **(B)** Lake Kaivoslampi (Finland), a humic boreal lake from which strain UB-Kaiv-W7 was obtained. **(C)** View from a cliff on lake Unterer Klaffersee, a clear water lake located in the Austrian Alps above the tree line at an altitude of 2116 m, from which strain MWH-UK1W16 was isolated.

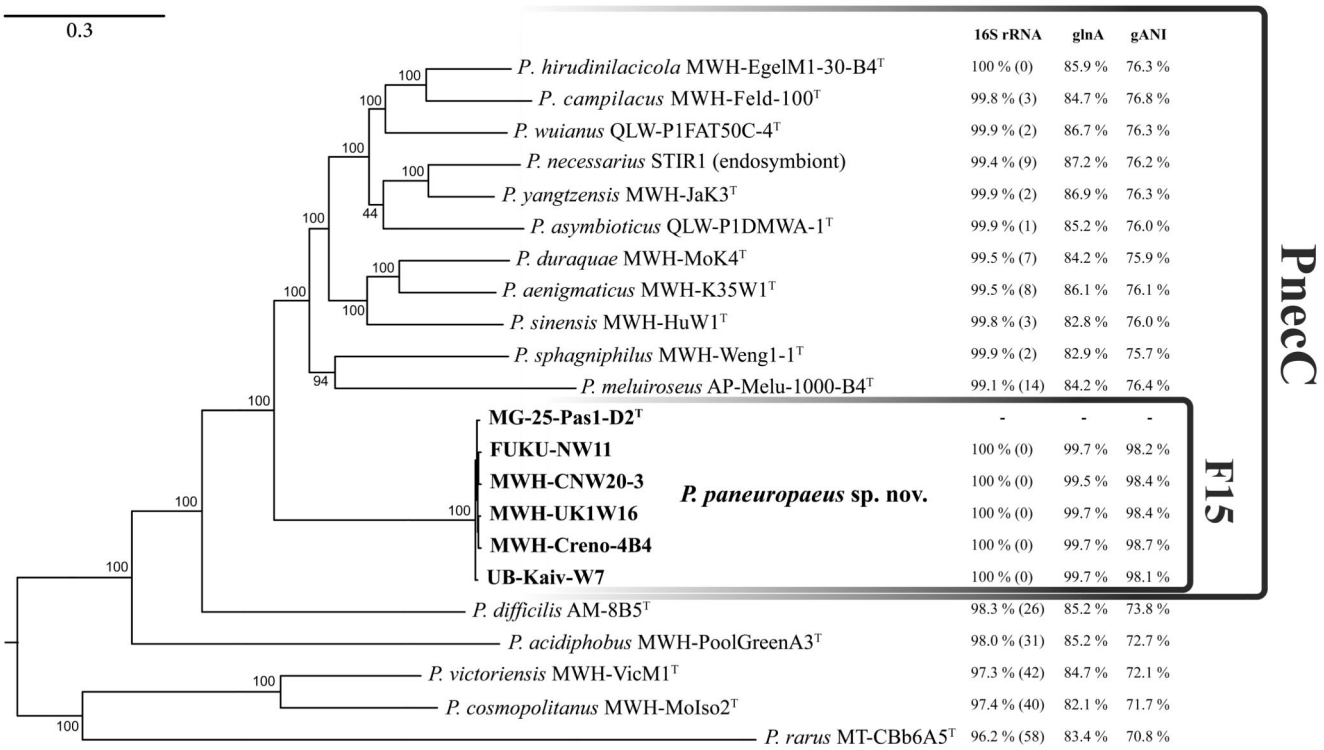

Fig. 2.

Reconstruction of the phylogenetic position of the six investigated *Polynucleobacter* strains. Bootstrapped RAxML tree calculated with nucleotide sequences of 319 shared genes. Bootstrap values (100 resamplings) are shown except for nodes within taxon F15, however, all those intra F15 nodes were supported by values ≥ 99%. Percentage values behind the strain names indicate whole length 16S rRNA gene sequence similarity (number of nucleotide mismatches are given in brackets), sequence similarity of partial (603 bp) glutamine synthetase genes, and gANI values obtained in pairwise comparisons of whole genome sequences. All data represent pairwise comparisons with strain MG-25-Pas1-D2^T. The tree was rooted with sequences of *Cupriavidus metallidurans* CH34^T (not shown, accession number: CP000352-CP000355 [45]). Accession numbers of genomes of PnecC strains are given in Table 2. Accession numbers of genomes of the other *Polynucleobacter* species are CP023276, CP023277, NJGG00000000, NTGB00000000, and FYEX00000000. Scale bar: 0.3 substitutions per nucleotide position.

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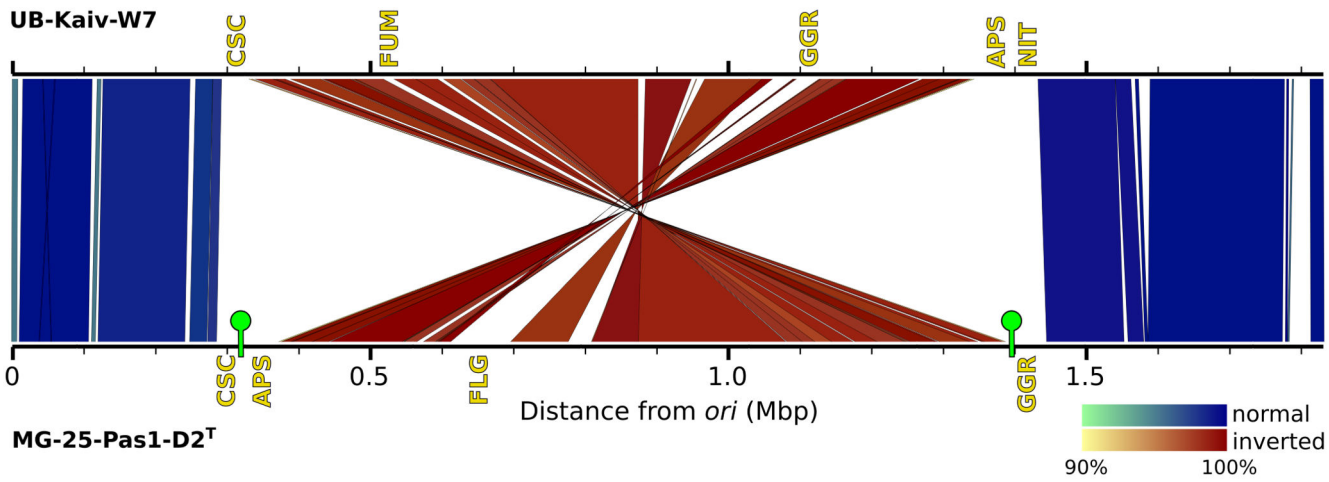

Fig. 3.

Whole genome alignment of strains UB-Kaiv-W7 and MG-25-Pas1-D2^T showing the chromosomal inversion. An (incomplete) selection of gene clusters present in genomic islands is labelled according to function as suggested by gene annotation (compare with the description of genomic islands of *Polynucleobacter asymbioticus* in [46]). APS, anoxygenic photosynthesis; CSC, cell surface composition; FLG, flagellum synthesis; FUM, fumarate reduction; GGR, giant gene region; NIT, nitrate assimilation. The differing gene content of strains UB-Kaiv-W7 and MG-25-Pas1-D2^T compared to the other F15 strains listed in Table 3 relates to the gene clusters APS, FLG, FUM, and NIT. The green pins indicate the positions of the two transposases, which may represent the sites of intrachromosomal recombination that likely happened in an ancestor of strain MG-25-Pas1-D2^T.

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Table 1

Lakes from which the six investigated *P. paneuropaeus* sp. nov. strains were isolated. Habitats are sorted from North to South. Observed ranges of pH and conductivity (Cond.) values are given if habitats were sampled repeatedly.

Strain	Lake	Country	Latitude (°N)	Longitude (°E)	Altitude (m)	pH	Cond. (µS cm^{-1})
MG-25-Pas1-D2^T (=DSM 103454^T)	Russevatn	Norway	69.445747	29.902438	45	5.5 - 5.9	15.4 - 28.8
UB-Kaiv-W7	Kaivoslampi	Finland	63.335417	28.832056	180	4.6 - 5.1	15.9 - 18.1
FUKU-NW11 (=DSM 103469)	Große Fuchskuhle, NW section	Germany	53.105646	12.984723	61	5.4	25.2
MWH-CNW20-3 (=DSM 103490)	Černé jezero	Czech Republic	49.179435	13.182309	1011	4.8	22.1
MWH-UK1W16 (=DSM 24086)	Unterer Klaffersee	Austria	47.300073	13.793077	2116	7.4 -7.5	21.9 - 27.7
MWH-Creno-4B4 (=DSM 103504)	Lac de Creno/Crena	France	42.204910	8.945997	1321	6.6 - 7.5	40.0 - 55.4

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Table 2

Genome characteristics of *Polynucleobacter* type strains and an endosymbiotic strain affiliated with the species *P. necessarius*. Only taxa belonging to subcluster PnecC are shown. Data on the *Polynucleobacter* type strains not affiliated with this subcluster can be found elsewhere [16, 28]. FL, free-living; E, endosymbiotic.

Species	Strain	*Polynucleo-bacter* subcluster	Life-style	Genome size (Mbp)	Scaf-folds	G+C content (mol%)	DDBJ/EMBL/GenBank accession number	IMG Genome ID	Reference
P. paneuropaeus sp. nov.	MG-25-Pas1-D2^T (=DSM 103454^T)	PnecC, F15	FL	1.83	1	45.6	CP030085	2770939595	This study
P. paneuropaeus sp. nov.	MWH-CNW20-3 (=DSM 103490)	PnecC, F15	FL	1.72	1	45.9	CP030087	2770939599	This study
P. paneuropaeus sp. nov.	MWH-Creno-4B4 (=DSM 103504)	PnecC, F15	FL	1.61	1	46.1	CP030086	2770939593	This study
P. paneuropaeus sp. nov.	UB-Kaiv-W7	PnecC, F15	FL	1.83	1	45.6	CP030088	2770939596	This study
P. paneuropaeus sp. nov.	FUKU-NW11 (=DSM 103469)	PnecC, F15	FL	1.79	14	45.8	QMCH00000000	2770939597	This study
P. paneuropaeus sp. nov.	MWH-UK1W16 (=DSM 24086)	PnecC, F15	FL	1.79	7	45.9	QMCG00000000	2770939594	This study
P. aenigmaticus	MWH-K35K1^T (=DSM 24006^T)	PnecC	FL	2.14	37	46.0	NGUO00000000	2675903126	[24]
P. asymbioticus	QLW-P1DMWA-1^T (=DSM 18221^T)	PnecC	FL	2.16	1	44.8	CP000655	640427129	[43]
P. campilacus	MWH-Feld-100^T (=DSM 24007^T)	PnecC	FL	1.98	9	45.2	NGUP00000000	2590828843	[42]
P. duraquae	MWH-MoK4^T (=DSM 21495^T)	PnecC	FL	2.03	1	45.2	CP007501	2634166443	[3]
P. hirudinilacicola	MWH-EgelM1-30-B4^T (=DSM 23911^T)	PnecC	FL	2.01	5	45.3	NAIA00000000	2590828818	[42]
P. meluiroseus	AP-Melu-1000-B4^T (=DSM 103591^T)	PnecC	FL	1.89	11	46.6	OANS00000000	2710724120	[28]
P. necessarius	STIR1 [host, *Euplotes aediculatus*]	PnecC	E	1.56	1	45.6	CP001010	2503982034	[44]
P. sinensis	MWH-HuW1^T (=DSM 21492^T)	PnecC	FL	2.32	19	45.5	LOJJ01000000	2630969031	[3]
P. sphagniphilus	MWH-Weng1-1^T (=DSM 24018^T)	PnecC	FL	2.04	17	45.6	MPIY01000000	2574179738	[25]
P. wuianus	QLW-P1FAT50C-4^T (=DSM 24008^T)	PnecC	FL	2.23	1	44.9	CP015922	2687453598	[23]
P. yangtzensis	MWH-JaK3^T (=DSM 21493^T)	PnecC	FL	2.05	42	45.4	LOJI01000000	2608642177	[3]

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Table 3

Presence and absence of selected genes in six *P. paneuropaeus* sp. nov. strains and in type strains of all other *Polynucleobacter* strains affiliated with subcluster PnecC. 1, *P. paneuropaeus* sp. nov. MG-25-Pas1-D2^{T}; 2, *P. paneuropaeus* sp. nov. MWH-Creno-4B4; 3, *P. paneuropaeus* sp. nov. MWH-UK1W16; 4, *P. paneuropaeus* sp. nov. MWH-CNW20-3; 5, *P. paneuropaeus* sp. nov. UB-Kaiv-W7; 6, *P. paneuropaeus* sp. nov. FUKU-NW11; 7, *P. hirudinilacicola* MWH-EgelM1-30-B4^{T}; 8, *P. campilacus* MWH-Feld-100^{T}; 9, *P. meluiroseus* AP-Melu-1000-B4^{T}; 10, *P. aenigmaticus* MWH-K35W1^{T}; 11, *P. sphagniphilus* MWH-Weng1-1^{T}; 12, *P. wuianus* QLW-P1FAT50C-4^{T}; 13, *P. asymbioticus* QLW-P1DMWA-1^{T}; 14, *P. duraquae* MWH-MoK4^{T}; 15, *P. sinensis* MWH-HuW1^{T}; 16, *P. yangtzensis* MWH-JaK3^{T}. ++, two gene clusters present; +, gene(s) present; -, gene(s) absent.

Genes putatively encoding	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Inorganic nutrients																
ABC-type Fe^{3+} transport system	-	-	-	-	-	-	+	+	-	+	-	-	-	+	+	+
feoAB genes (uptake of Fe^{2+})	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	+
ABC-type iron complex transport system	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-
ABC-type Nitrate/Nitrite/Cyanate transporter	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	+
Nitrate reductase (assimilatory)	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	+
Nitrite reductase (assimilatory)	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	+
Cyanate lyase (releases NH_3 a. CO_2 from cyanate)	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	+
Urease and ABC-type urea transporter	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
Oxidative phosphorylation/ Energy metabolism																
Cytochrome bd-I terminal oxidase (CydAB)	+	+	+	+	+	+	-	-	-	-	+	+	+	-	+	-
Fumarate reductase	-	-	-	-	+	-	+	+	-	+	+	+	-	+	-	+
Carbon monoxide dehydrogenase	-	-	-	-	-	-	-	-	-	+	+	+	-	++	-	+
Acetate permease actP	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-
Light energy conversion																
Anoxygenic photosynthesis gene cluster	+	-	+	+	+	+	+	+	-	-	-	+	-	+	-	-
Proteorhodopsin gene	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Motility																
Flagellum gene cluster	+	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-

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Table 4

Phenotypic characteristics of three *P. paneuropaeus* sp. nov. strains and all type strains affiliated with subcluster PnecC of the genus *Polynucleobacter*. All thirteen strains shared the ability to assimilated acetate. 1, *P. paneuropaeus* MG-25-Pas1-D2^T; 2, *P. paneuropaeus* MWH-Creno-4B4; 3, *P. paneuropaeus* MWH-UK1W16; 4, *P. hirudinilacicola* MWH-EgelM1-30-B4^T; 5, *P. campilacus* MWH-Feld-100^T; 6, *P. meluiroseus* AP-Melu-1000-B4^T; 7, *P. aenigmaticus* MWH-K35W1^T; 8, *P. sphagniphilus* MWH-Weng1-1^T; 9, *P. wuianus* QLW-P1FAT50C-4^T; 10, *P. asymbioticus* QLW-P1DMWA-1^T; 11, *P. duraquae* MWH-MoK4^T; 12, *P. sinensis* MWH-HuW1^T; 13, *P. yangtzensis* MWH-JaK3^T. +, increase in optical density (OD); w, weak increase in OD; -, no significant increase in OD. Data for columns 4 and 5 were taken from [42], data for columns 6-13 from [28]. All presented data were obtained in the same lab under standardized conditions.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Cell morphology	short rods	short rods	short rods	short rods	slightly curved rods	rods	short rods	short rods	short rods	short rods	curved rods	short curved rods	short rods
Cell length (µm)	0.6 - 1.2	0.4 - 0.9	0.4 - 1.2	0.5 - 1.2	0.5 - 2.4	0.8 - 2.1	0.5 - 1.0	0.6 - 1.0	0.6 - 1.7	0.7 - 1.2	0.9 - 2.9	0.6 - 1.4	0.5 - 1.5
Cell width (µm)	0.3 - 0.6	0.3 - 0.5	0.3 - 0.6	0.3 - 0.5	0.3 - 0.7	0.3 - 0.6	0.3 - 0.5	0.3 - 0.5	0.3 - 0.6	0.4 - 0.5	0.4 - 0.5	0.4 - 0.5	0.3 - 0.5
Temperature range of growth (°C)	5 - 31	5 (w) - 32	5 - 32	5 - 31	5 - 28	5 - 28 (w)	5 - 32 (w)	5 - 31	5 - 34	5 - 34 (w)	5 - 30	5 - 35	5 - 35
NaCl tolerance (%NaCl, w/v)	0 - 0.5 (w)	0 - 0.4	0 - 0.4	0 - 0.1	0 - 0.4 (w)	0 - 0.4 (w)	0 - 0.3	0 - 0.4	0 - 0.5	0 - 0.5 (w)	0 - 0.3	0 - 0.5	0 - 0.3 (w)
Anaerobic growth	-	-	w	-	-	-	-	-	-	+	-	-	+
Assimilation of:													
Glyoxylate	+	+	+	-	-	-	-	-	w	w	-	-	-
Glycolate	+	+	+	-	+	-	-	-	-	-	-	-	-
Propionate	w	-	w	-	-	+	w	w	+	+	-	+	+
Pyruvate	+	+	+	+	w	+	+	+	+	+	+	+	+
Malonate	w	+	w	-	w	w	+	+	w	w	-	+	w
Oxaloacetate	+	+	+	+	+	+	-	-	+	-	+	+	+
Malate	+	+	+	-	-	+	+	+	+	+	w	+	+
Fumarate	+	+	+	w	-	+	+	+	+	+	w	+	+
Succinate	+	+	+	+	-	+	+	+	+	+	+	+	+
Oxalate	+	-	-	+	-	-	w	-	-	-	-	-	-
Citrate	w	-	-	-	-	-	-	-	-	-	-	-	-
Levulinate	w	w	+	-	-	w	-	w	w	w	-	-	-
D-Galacturonate	-	-	+	-	-	-	w	+	w	w	w	w	w

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	1	2	3	4	5	6	7	8	9	10	11	12	13
D-Mannose	w	w	w	w	w	-	-	w	-	w	-	-	-
D-Glucose	-	-	-	-	-	-	w	-	-	w	w	-	-
D-Galactose	-	-	-	-	-	-	-	-	-	w	-	-	-
D-Lyxose	-	w	-	-	-	-	w	-	-	w	w	-	w
D-Fructose	+	w	-	-	-	-	-	w	w	w	w	-	w
L-Fucose	-	-	-	w	-	-	-	w	-	w	-	-	w
D-Sorbitole	-	-	-	-	+	w	-	w	-	w	-	-	-
L-Glutamate	w	-	w	w	-	+	+	+	+	+	-	+	-
L-Histidine	-	-	-	-	-	-	-	-	+	-	w	-	-
L-Aspartate	-	-	-	-	-	w	-	-	+	+	-	-	-
L-Cysteine	+	+	+	+	-	+	+	+	w	+	+	w	+
L-Alanine	-	-	-	+	-	-	+	+	+	w	-	-	-
L-Asparagine	-	-	-	-	-	-	-	-	w	w	-	-	-
L-Leucine	-	-	-	-	w	-	-	-	-	-	w	-	w
L-Serine	-	-	-	-	-	-	w	-	-	-	-	-	-
Betaine	-	-	-	-	w	-	-	-	-	w	-	-	-

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Table 5

Major fatty acid compositions of three *Polynucleobacter panaeuropaeus* sp. nov. strains and all type strains affiliated with subcluster PnecC of the genus *Polynucleobacter*. 1, *P. paneuropaeus* MG-25-Pas1-D2^T; 2, *P. paneuropaeus* MWH-Creno-4B4; 3, *P. paneuropaeus* MWH-UK1W16; 4, *P. hirudinilacicola* MWH-EgelM1-30-B4^T; 5, *P. campilacus* MWH-Feld-100^T; 6, *P. meluiroseus* AP-Melu-1000-B4^T; 7, *P. aenigmaticus* MWH-K35W1^T; 8, *P. sphagniphilus* MWH-Weng1-1^T; 9, *P. wuianus* QLW-P1FAT50C-4^T; 10, *P. asymbioticus* QLW-P1DMWA-1^T; 11, *P. duraquae* MWH-MoK4^T; 12, *P. sinensis* MWH-HuW1^T; 13, *P. yangtzensis* MWH-JaK3^T. Compounds at a percentage of 0.2 or higher are listed. Data for columns 4 and 5 were taken from [42], data for columns 6-13 from [28].

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
$C_{10:0}$	-	-	-	-	3.1	-	-	-	-	-	-	-	-
$C_{12:0}$	4.0	4.0	4.1	6.8	3.6	4.8	3.4	4.4	4.0	3.9	4.3	4.7	4.1
$C_{14:0}$	0.4	0.3	0.4	0.6	0.8	-	0.5	1.4	0.2	0.6	0.5	0.4	1.0
$C_{15:0}$	-	-	-	-	0.3	-	-	-	-	0.3	-	-	-
$C_{16:0}$	28.5	20.0	41.7	26.3	38.9	30.0	18.0	26.2	18.5	25.5	16.8	27.5	19.9
$C_{17:0}$	-	-	-	-	0.4	-	0.6	-	-	-	-	-	-
$C_{18:0}$	1.2	1.1	2.3	0.7	1.0	1.0	1.8	0.9	0.9	0.7	0.4	1.3	0.6
$C_{20:0}$	-	-	-	-	-	-	-	-	-	0.7	-	-	-
$C_{14:1}$ ω5c	-	-	-	-	0.3	-	-	0.5	-	-	-	-	-
$C_{16:1}$ ω5c	-	-	-	-	-	-	-	0.4	-	-	-	-	0.4
$C_{16:1}$ ω7c	31.2	31.8	34.6	35.7	40.1	36.9	35.9	35.8	39.1	39.6	41.3	36.9	35.6
$C_{18:1}$ ω7c	16.9	26.8	3.8	15.7	0.5	10.5	19.2	15.1	27.8	15.6	18.9	14.1	20.6
11-methyl $C_{18:1}$ ω7c	4.6	3.0	0.6	3.3	-	4.2	7.9	4.3	2.7	2.6	4.5	3.1	6.4
$C_{12:0}$ 2-OH	1.6	2.3	2.4	0.3	-	1.3	-	1.9	1.0	1.8	0.6	1.3	1.9
$C_{16:1}$ 2-OH	0.8	-	-	-	-	-	2.2	0.5	0.5	-	2.0	-	0.3
$C_{16:0}$ 3-OH	0.3	0.5	0.6	-	-	-	-	-	-	-	-	-	-
Feature 2	10.2	9.8	9.5	10.2	11.0	10.2	9.8	8.7	4.7	8.6	9.8	10.9	8.0
Feature 7	-	-	-	0.5	-	1.0	0.3	-	0.4	-	0.8	-	1.0

Summed features represent groups of two fatty acids which could not be separated by GLC and the MIDI system, such as summed feature 2 containing $C_{16:1}$ isoI and $C_{14:0}$-3OH and summed feature 7 containing $C_{19:1}$ ω6c and an unknown compound with an ECL of 18.846.